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Medical Defense Against Protein Toxin Weapons

Review and Perspective

Charles B. Millard

1. PROTEIN TOXIN WEAPONS

The term "toxin weapon" has been used to describe poisons, classically of natural origin but increasingly accessible by modern synthetic methods, which are suitable for delivery on a battlefield in a form that causes death or severe incapacitation at relatively low concentrations (reviewed in ref. *1*). Several of the most important toxin weapons are proteins, and these molecules are the focus of this chapter. Recent technological changes have increased the importance of protein toxins for biological warfare (BW): (a) progress in biotechnology has made large-scale production and purification feasible for a larger number of protein toxins; (b) molecular biology techniques, especially the polymerase chain reaction, have enabled the identification, isolation and comparison of extended families of previously obscure natural toxins; and (c) gene manipulation and microbiology have greatly expanded the accessible delivery vehicles for protein toxins to include, for example, natural or genetically modified bacteria and engineered viruses.

Advances in biotechnology notwithstanding, if we consider only those protein toxins with characteristics suitable for direct use as mass-casualty weapons in the absence of replicating, biological delivery systems, then only a small subset of known proteins are of immediate concern *(1)*. The list of practicable toxin weapons is small because: (a) proteins are not volatile and generally do not persist long in the environment; (b) simple, physical protection offers an effective natural defense against foreign proteins; and (c) relatively sophisticated research, development, testing, and evaluation is required to establish conclusively that each specific protein toxin is a viable open-air, aerosol weapon.

Although small in number, toxin weapons should not be neglected. Similarly to chemical weapons or noninfectious biological agents such as anthrax spores, toxins offer the aggressor a tactical weapon to strike at the enemy in a controlled manner that is difficult or impossible with infectious agents, for example by the selective contamination of key terrain or high-value targets. Aerosolized protein toxins can be used both as lethal agents and as severe incapacitating agents, thereby greatly burdening medical

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14. ABSTRACT

The term "toxin weapon" has been used to describe poisons, classically of natural origin but increasingly accessible by modern synthetic methods, which are suitable for delivery on a battlefield in a form that causes death or severe incapacitation at relatively low concentrations. Several of the most important toxin weapons are proteins, and these molecules are the focus of this chapter. Recent technological changes have increased the importance of protein toxins for biological warfare (BW): (1) progress in biotechnology has made large-scale production and purification feasible for a larger number of protein toxins; (2) molecular biology techniques, especially the polymerase chain reaction, have enabled the identification, isolation and comparison of extended families of previously obscure natural toxins; and (3) gene manipulation and microbiology have greatly expanded the accessible delivery vehicles for protein toxins to include, for example, natural or genetically modified bacteria and engineered viruses.

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care and logistical systems. Moreover, unlike chemical nerve agents and anthrax spores, there are no effective postexposure treatments widely available for the most dangerous protein toxins.

In what forms can we expect to encounter protein toxin weapons? Most predictions of the potential medical threat posed by direct delivery of protein toxin weapons are predicated on military doctrine that assumes a trained, equipped, and healthy population in control of its own food and water supplies. For a prepared military force, the primary threat in an open-air battlefield environment is stable, respirable aerosols of the most toxic molecules by weight (reviewed in ref. *1*). Closed-air delivery of respirable aerosols, for example, within a building or other enclosed space, poses a secondary threat that would be expected to cause far fewer casualties but may expand the set of potential toxin weapons to include those with lower toxicity or those lacking outdoor stability.

An accurate assessment of which protein toxins are effectively "most toxic" by aerosol delivery must consider complex biological and environmental variables. As a first approximation, the most potent toxins (typically bacterial proteins) are those that are lethal for 50% of test animals (i.e., toxin LD_{50}) at amounts less than 25 ng/kg by intravenous or intra-peritoneal exposure routes *(1–4)*. Aerosol lethality is a function of both toxin concentration and exposure time, and this is reported as an LCt_{50} value with units of $mg/min/m^{-3}$.[1] Direct comparison among published toxin LD_{50} or LCt_{50} values can be easily confounded by numerous experimental variables, including the method of aerosol exposure, time of exposure, breathing patterns, and other interspecies variations among animal models, as well as physical differences in the purity, stability, or potency of the toxin employed. Furthermore, lethality data alone are unsatisfactory for gaging the severe incapacitation caused by lung injury that may be enhanced by aerosol delivery routes for proinflammatory toxins.

Some bacterial protein toxins are notoriously potent food poisons *(5)*. Sabotage of food or beverage supplies with protein toxins is unlikely to produce mass casualties against a military force but could have a significant disruptive effect upon unprepared populations. Fortunately, the threat of intentional poisoning is significantly mitigated by modern food-processing practices, dilution, and routine public health measures such as monitoring, rapid communication, and other controls.

Protein toxins are not expected to pose a significant mass-casualty threat by percutaneous or ocular delivery routes because the stratified epithelial tissues of skin and cornea provide barriers that limit penetration of foreign proteins, provided that the tissue has not been compromised by injury or other means. Protein toxins may cause incapacitating ocular inflammation by direct or indirect effects on exposed cornea and conjunctiva, but these effects generally are reversible.

From even a brief assessment of the medical threat posed by protein toxin weapons, it is apparent that respiratory protective equipment, for example, a gas mask or respirator, and immediate decontamination offer the best defense. However, if natural or other physical barriers and rapid decontamination fail to prevent internalization of a protein toxin, then survival may depend on the availability of adequate medical countermea-

[1] A detailed discussion of the pathogenesis of toxin bioaersols, including selected toxin aerosol LCt_{50} values, is presented elsewhere in this volume by Pitt and LeClaire.

sures, including vaccines, other pretreatments, or antidotes. The purpose of this chapter is to provide a review of selected medical products under development for protection against protein toxins of military significance and to offer a perspective on the critical role of protein engineering[2] in the iterative process of optimizing those medical products. In addition to medical countermeasures, the status of protein engineering of toxins also is discussed.

2. OVERVIEW OF MEDICAL COUNTERMEASURES

2.1. Vaccines

Vaccination, the intentional induction of a lasting, protective immune response mediated by antibodies, offers one of the most powerful, flexible, and safe methods known to achieve medical protection from protein toxins. Ideal vaccines to protect against BW protein toxins would safely induce lasting levels of high-avidity IgG antibodies within the alveolar lining fluid, as well as IgA antibodies secreted into the mucous membranes lining the lung airway, such that toxins are neutralized before reaching their biological targets.

There is a general, progressive strategy that has been employed for developing vaccines to protect against protein toxin threats. Initially, an inactivated "toxoid" vaccine is prepared from biological homogenates or crude toxin preparations. Toxoid vaccines provide a generally safe and effective solution, as exemplified by the enduring use of tetanus toxoid vaccines worldwide *(6)*. Yet, toxoid vaccines are susceptible to production, safety, or storage limitations that stem from use of denaturants, crosslinking agents, residual live toxin, or from the partial reversion of inactivated toxoid back to active toxin. To improve on toxoid vaccines, effective "neutralizing epitopes" are identified within toxin structures, and this information is applied to develop recombinant immunogens with enhanced safety and ease of production.

Protein engineering, especially recombinant DNA technology for site-specific substitutions and commercial protein expression systems, has contributed to the development of a number of new, recombinant vaccine candidates that were inaccessible previously because of small quantities or inherent toxicity of natural immunogens. Recombinant immunogens are generally well-defined and suited to current Good Manufacturing Practices. However, the limited immune system responses to purified, recombinant proteins or polypeptide subunits, compared to responses to natural infections or attenuated vaccines, must be overcome by careful selection of epitopes and the use of adjuvants or other activators *(7)*.

As a more-detailed structural concept of the toxin and vaccine candidate emerges, protein engineering methods may permit cycles of vaccine improvement, for example, to achieve better presentation of the neutralizing epitopes or enhanced stability. Engineering may result in production of new immunogens based on inactivated holotoxins, polypeptide subunits, or independent polypeptide domains.

Because of the importance of the aerosol threat in BW, traditional vaccination strategies for some toxins may benefit from additional protection of critical target organs,

[2]Protein engineering is the deliberate modification of polypeptide structure to achieve a desired form or function.

especially the portals of toxin entry such as the respiratory tract. Alternate vaccine-delivery methods, including transdermal, intranasal, inhalation, or oral routes for vaccine priming or boosting, may permit more effective administration of engineered vaccines to induce stronger mucosal antibody responses. This approach includes the application of novel devices or adjuvant-device combinations that also may facilitate simultaneous delivery of multiple immunogens *(8)*. Along with nontraditional routes of vaccine delivery, it may be possible to enhance protection of target organs by the use of adjunct therapeutics.

Despite the power of vaccination to protect against protein toxin weapons, there will continue to be a limited number of military or first responder scenarios in which there is insufficient time for vaccines to elicit a protective immune response. Additionally, it may be impractical or undesirable to vaccinate large, healthy populations against the relatively remote threat of all potential toxins. The vaccine candidates currently available have been developed for use in limited, volunteer military forces comprising mostly healthy young adults subject to regular medical screening and care. Additional optimization studies may be required to ensure safe administration to larger, more diverse civilian populations.

Other limitations may arise with complete reliance on vaccination as a medical solution for toxin weapons. As the clinical use of toxins themselves as medical therapies (so-called "medicotoxins") continues to expand, for example, the growing medical use of botulinum neurotoxin (BoNT) injections to control the cholinergic neuromuscular junction in various disease states (reviewed in ref. *9*), it will become increasingly difficult to justify the use of vaccination against toxin weapons for the general population. Once an individual is vaccinated against a toxin, the clinical administration of that specific molecule as a medicotoxin becomes much more difficult *(10)*.

Consequently, the development of antitoxin therapeutics as adjuncts to vaccines and, in some BW scenarios, as viable replacements for vaccination is an important component of medical defense against protein toxins. Therapeutic approaches include antibody-based biological therapeutics, as well as emerging biomedical research to discover cost-effective small-molecule antidotes.

2.2. *Immunotherapeutics*

The use of antibody molecules before an anticipated toxin exposure, or as a therapy immediately after exposure, is called "passive immunotherapy" or "antitoxin" therapy. Specific antibodies or processed binding fragments of antibodies (FAbs) can be prepared by various technologies, including vaccination of a suitable donor with toxin vaccine candidates or recombinant DNA-based protein expression systems. Purified immunotherapeutics subsequently can be administered to at-risk or exposed recipients as "bioscavengers" to bind and eliminate toxic molecules from the body before they reach critical target sites. The use of preformed antibodies to mitigate symptoms of BoNT toxin, for example, has been an accepted part of the routine clinical management of food-borne botulism in humans since the 1960s *(11)*.

Antitoxin antibodies historically have been captured from the polyvalent immune serum of vaccinated or hyperimmune animals and subsequently used to treat human patients exposed to protein toxins. Although processing to remove expendable portions of the antibody molecule that are distal to the essential antigen combining end

("despeciation") may reduce the human immune reaction, serious side effects such as anaphylaxis and serum sickness still may occur because the animal-derived products are recognized as foreign. Because of this immunological "rejection" of foreign protein by the human patient, animal-derived products become more dangerous if administered repeatedly; this greatly limits the utility of animal products as a pretreatment.

Antitoxin derived from human immune serum of vaccinated volunteers overcomes the major limitations imposed by immunological rejection of animal serum. For example, human antitoxin products would be expected to circulate longer and could be given by repeated injections, thus opening up the possibility of a safe pretreatment for toxin exposure. However, the production of suitable human immune serum in sufficient quantities for use in mass casualty scenarios may be impractical because the number of suitable immune human donors is small. Furthermore, the widespread use of human immune serum antitoxin carries the risk of transfer of unknown or undetectable human pathogens or adventitious agents from donor to recipient.

Although the flexibility and specificity of an antibody-based therapeutic is unquestionable, the application of this approach as a routine medical solution in a field situation poses several logistical and technical challenges: (a) the success depends critically on stability of the proper three-dimensional structure of the antibody therapeutic employed; (b) the therapeutic window for antibody use is narrow because symptoms of toxin exposure typically appear hours to days after exposure when the toxin already has bound its target or has been internalized to intracellular compartments inaccessible to antibody molecules; and (c) antibodies and FAbs are large molecules that bind reversibly with a limited stoichiometry and, therefore, it generally will require large amounts of therapeutic by weight to neutralize supralethal quantities of toxin.

2.3. Small-Molecule Experimental Therapeutics

Selective, low-molecular-weight drugs are unavailable at present for the most deadly protein toxin weapons, but active research programs are underway and have produced key resources in the past several years, including solved three-dimensional X-ray structures of toxin and toxin-inhibitor complexes, cloned toxin subunit genes, and specific, high-throughput toxin activity assays. In addition to antibody-based scavenger approaches, novel toxin therapeutics may be directed against one or more of the molecular steps required for intoxication. For those protein toxins that achieve very high potency by enzymatic catalysis, for example, it may be possible to develop selective, very high affinity or irreversible active-site inhibitors as effective toxin therapeutics.

In summary, four general approaches are being taken to develop pharmaceuticals to protect against protein toxin weapons: toxoid vaccines, engineered vaccines, immunotherapeutics, and small-molecule therapeutics. I will expand on the relative advantages and disadvantages of each approach through a review of past and ongoing research efforts to protect against three specific protein toxin weapons: BoNT, *Staphylococcus aureus* enterotoxins (SE), and ricin toxin from *Ricinus communis*. BoNT, SE, and ricin are chosen on the basis of the maturity of medical product candidates currently under development and also because each represents an important class of protein toxin: cholinergic toxins, immune system modulators, and ribosome inactivating toxins, respectively.

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3. BoNT

Botulism is caused by a family of potent neurotoxins (BoNT) that are produced for unknown reasons by *Clostridium botulinum* bacteria from one of at least seven different serotypes (designated BoNT types /A through /G) *(12)*. Four of the serotypes (/A, /B, /E, and, less commonly, /F) are significant for human poisoning through contaminated food, wound infection, or infant botulism (reviewed in ref. *13*). Although botulism is a relatively rare disease worldwide, the extreme toxicity of BoNT makes it a potential toxin weapon *(11,14)*.

Within the past few years, three-dimensional structures of holotoxins or isolated domains of toxins from *Clostridium* bacteria have been solved, and consequently, a more complete picture of toxin function is emerging *(15–18)*. Like the closely related tetanus neurotoxin (TeNT), the BoNT proteins are disulfide-bonded heterodimers composed of an approx 50 kD zinc metalloprotease "light chain" and an approx100 kD receptor-binding "heavy chain" (Hc). The Hc has been subdivided structurally and functionally into a C-terminal domain that binds the toxin to gangliosides and other receptors on the surface of peripheral cholinergic neurons (so-called Hc domain), and an N-terminal domain that is believed to enhance cell binding and translocation of the catalytic light chain across the vesicular membrane (reviewed in ref. *19; see* Fig. 1). Additionally, BoNT naturally is associated with numerous nontoxic "accessory proteins," some of which may stabilize the toxins in vivo *(20)*.

The mechanism by which BoNT traverses neuron cell membranes is incompletely understood, but it may involve a large conformational change in the toxin. A conformational change or partial unfolding of the light chain has been proposed to explain passage of the toxin catalytic portion through narrow transmembrane channels or pores formed by the amino terminal portion of the BoNT heavy chain *(21–24)*.

Once inside the neuron, the catalytic subunit of BoNT acts as a selective, zinc metalloprotease to cleave essential polypeptide components of the so-called "SNARE complex" required for normal neurotransmitter release or membrane fusion. BoNT/A, /C1, and /E cleave the polypeptide SNAP-25 (BoNT/C1 cleaves syntaxin), and BoNT/B, /D, /F, and /G cleave synaptobrevin (reviewed in ref. *19*). The exact mechanisms by which the soluble *N*-ethyl maleimide-sensitive factor attachment protein receptors (SNARE) complex mediates vesicle fusion or release of neurotransmitter acetylcholine (ACh) into the synaptic cleft remain controversial, but it is clear that the integrity of the complex is critical for normal cholinergic nerve transmission (reviewed in refs. *25–27*).

By disrupting ACh exocytosis at the peripheral neuromuscular junction, BoNT causes cholinergic autonomic nervous system dysfunction in effected patients. Signs and symptoms of BoNT intoxication typically manifest 12–36 h after toxin exposure and include generalized weakness, lassitude, and dizziness. There may be decreased salivation and dry mouth or sore throat; motor symptoms reflect cranial nerve dysfunction, including dysarthria, dysphonia, and dysphagia, followed by symmetrical descending and progressive muscle paralysis *(13)*. Without adequate supportive care, death may occur abruptly as a result of respiratory failure. The molecular precision of BoNT renders it among the most toxic substances known by weight; internalized BoNT may cause fatal paralysis in animals at nanogram/kilogram levels *(3)*.

3.1. BoNT Toxoid Vaccines

Preparations of inactivated, partially purified BoNT have been used as vaccines to protect humans for many years *(28)*. BoNT from each of the serotypes /A–/E was prepared, inactivated with formalin, adsorbed to aluminum hydroxide, and blended to produce an effective pentavalent toxoid vaccine (PBT) *(29–33)*. The PBT vaccine currently is administered to at-risk laboratory workers under an Investigational New Drug (IND) protocol. An effective vaccination regimen has been found to comprise initial doses (0.5 mL) of 10 μg of toxin protein equivalent given at 0, 2, and 12 wk, followed by annual boosters. Annual boosters have been offered contingent on serum-neutralizing BoNT antibody levels, as measured by mouse neutralization assays. In human volunteers, the available PBT induces antibodies that neutralize the toxicity of BoNT/A and /B in mouse bioassays *(34)*. Protective titers for other serotypes have not been established as rigorously as for BoNT/A, but neither have the exposure threshold levels of toxin for which a laboratory worker may be at risk.

Additional BoNT toxoid vaccines have been produced and used safely in humans. The PHLS Center for Applied Microbiology and Research, Porton Down (Salisbury, Wiltshire, England) produced a monovalent BoNT/A vaccine. A monovalent toxoid vaccine for BoNT/F subsequently was developed after BoNT/F botulism outbreaks were diagnosed in 1980–1990 *(35)*. A tetravalent vaccine candidate (BoNT/A, /B, /E, and /F) has recently been produced for human use *(36)*.

Despite the effectiveness of BoNT toxoid vaccines, there are significant cost and technical barriers associated with their production. Because of the sporulating nature of *C. botulinum*, a dedicated, contained manufacturing facility currently is required to produce toxoid. Additionally, the natural yields of BoNT from *C. botulinum* are low relative to the quantities of toxin needed for vaccine starting material. Moreover, there is a small but significant number of minor adverse reactions associated with toxoid vaccine, perhaps because of the use of formalin in the manufacturing process (reviewed in ref. *37*). These concerns have led to the development of recombinant BoNT vaccines *(38–40)*.

3.2. Engineered BoNT Vaccines

Simpson et al. reported that TeNT Hc fragments could compete for neuron binding and, thereby, antagonize the neuromuscular blocking properties of native TeNT and, to a lesser extent, BoNT *(41,42)*. This observation led to the immunization of mice against TeNT with fragments of TeNT synthesized in *E. coli (38,43)*. A similar vaccine for BoNT/A based on the recombinant Hc became possible once the toxin gene was cloned and expressed *(39,40,44)* (*see* Fig. 1). Subsequent epitope mapping of BoNT/A identified two specific polypeptides, both from Hc ($H_{455–661}$ and $H_{1150–1289}$), that were capable of protecting mice from a supralethal challenge with the toxin *(45)*.

The US Army Medical Research Institute of Infectious Diseases (USAMRIID) developed recombinant BoNT Hc vaccine candidates for BoNT/A, /B, and /F that confer protection in mice against supralethal challenges with toxin *(46–50)*. This approach recently was extended to include BoNT Hc fragments from BoNT/C and /D *(51)*. Unlike the BoNT toxoids, the recombinant Hc vaccine candidates do not require treatment with denaturants and are not susceptible to reversion of catalytic activity. If no

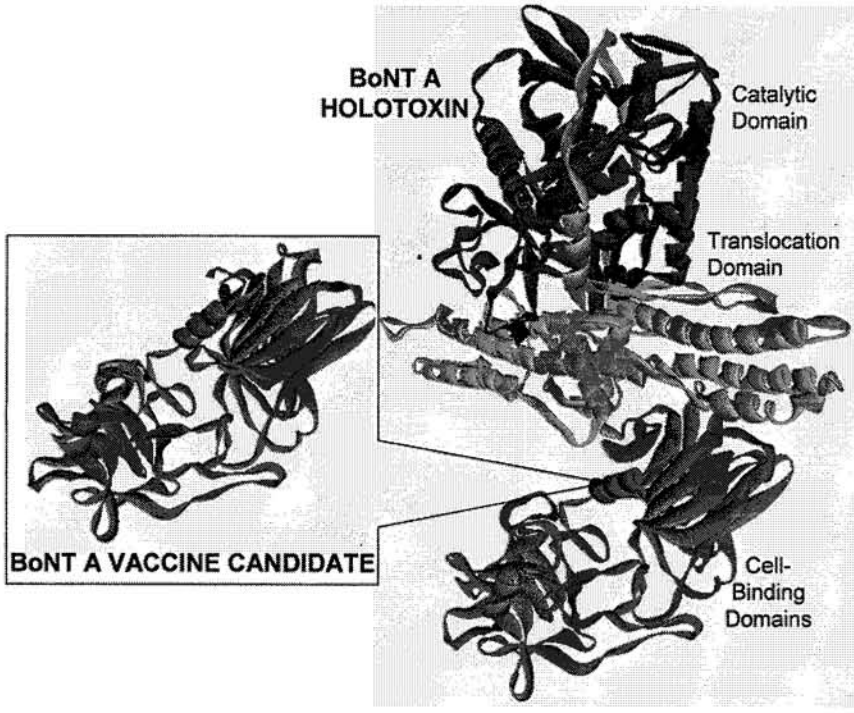

Fig. 1. Topology of recombinant BoNT Hc vaccine candidates with respect to the overall three-dimensional structure of the holotoxin. The figure shows a ribbon diagram of the BoNT/A holotoxin structure solved by X-ray crystallography (PDB entry 3BTA) that includes the three functional domains colored separately. A hypothetical projection of the recombinant vaccine candidate structure based upon the known amino acid sequence is shown as inset.

serious safety issues are identified, then the BoNT Hc vaccine candidates will require final formulation with an appropriate adjuvant, and optimization of the stability of each vaccine during scale-up production and formulation *(37,50,52)*. Additionally, a strategy for effective delivery of multiple recombinant BoNT Hc subunit immunogens is needed to ensure protection against all relevant BoNT serotypes.

Although apparently safe and effective as vaccine candidates, an inherent limitation of the recombinant Hc fragment vaccines is their lack of cross-reactivity among BoNT serotypes. A separate Hc fragment immunogen is required for each BoNT serotype and, perhaps, for some different strains of each BoNT serotypes. Future protein engineering studies may employ detailed structural comparisons of essential residues within the Hc binding sites among the relevant BoNT serotypes to identify conserved epitopes *(53)*. The solved X-ray crystal structures of receptor-binding domains from TeNT and multiple BoNT serotypes, both free and bound with receptor analogs, should facilitate this approach by identifying critical, conserved binding features among serotypes *(18,54)*. Additional work is needed to explore the possibility of developing new vaccine candidates based on cross-reactive neutralizing epitopes within the translocation and catalytic domains of different BoNT serotypes *(55)*.

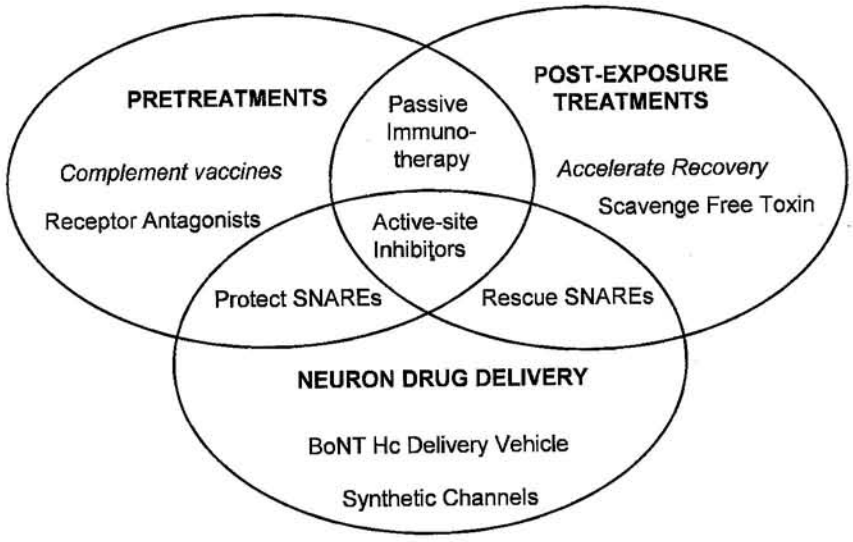

Fig. 2. Venn diagram depicting several complementary, mechanism-based research approaches being undertaken to develop novel therapeutics for protection against BoNT. Current therapeutics efforts include three primary research areas: (a) pretreatments; (b) postexposure antidotes; and (c) cholinergic neuron drug delivery systems for delivery therapeutics as appropriate.

3.3. *Alternate Delivery of BoNT Vaccines*

Several alternate vaccine-delivery routes for recombinant BoNT Hc immunogens have been explored recently in animal models including inhalation and oral vaccine delivery, as well as the use of self-replicating RNA virus or DNA-based vectors *(56–59)*. Proof-of-concept for the use of inactivated holotoxin as an oral immungen was reported by Simpson et al. *(60,61)*. However, it remains unproven whether these experimental delivery approaches offer any practical advantage for BW defense against BoNT compared with traditional, intramuscular vaccination. More data are needed describing the kinetics and biodistribution of BoNT after aerosol exposure in primates to evaluate whether there is a role for boosting mucosal immunity in protecting against supralethal BoNT exposures.

Protein engineering also may permit a combination of the toxoid and recombinant vaccine approaches. It has been shown that expression levels of BoNT can be increased in an *E. coli* system by amplifying specific transfer RNA (tRNA) genes for rare codons *(62)*. Additionally, progress also has been made on bacterial expression systems based on non-toxigenic strains of *C. botulinum (63)*. These results suggest the possibility that superior BoNT toxoid vaccines might be produced in *E. coli* or other protein expression systems by introducing active-site substitutions to selectively inactivate holotoxin, without the need for costly, dedicated production facilities or the risk of toxin reversion that limits older toxoid technology. Similarly, these tools may facilitate the future design of stable, multivalent, BoNT vaccines based on recombinant chimeras of multiple serotypes.

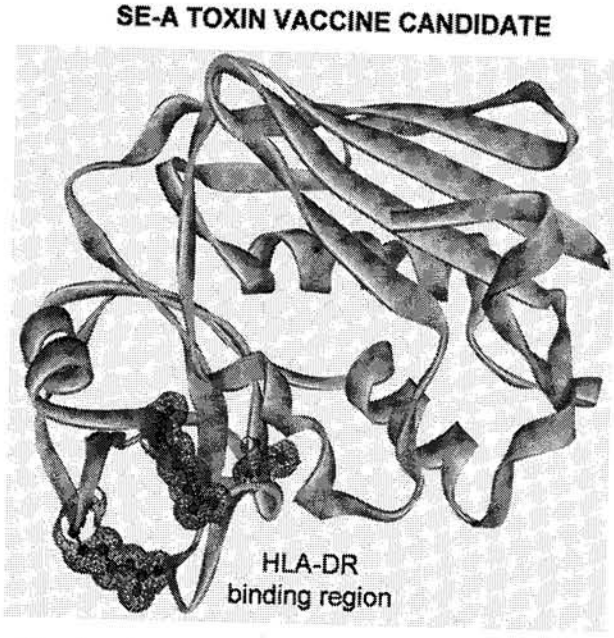

Fig. 3. Position of the three inactivating substitutions used to produce the current recombinant SE vaccine candidates with respect to the overall toxin structure and HLA binding site. The figure shows a ribbon diagram of the SE/A triple substitution structure solved by X-ray crystallography (PDB entry 1DYQ). The side chains of the substituted amino acid residues (Arg70, Arg48, and Ala92) are displayed with VDW surfaces.

3.4. *Immunotherapeutics*

Current medical treatment for BoNT intoxication is likely to involve prolonged life-support for incapacitated survivors, including the continual use of mechanical ventilation *(11)*. The potential of BoNT as a mass casualty weapon, combined with the high cost and logistical burden of symptomatic medical treatment, has led to increased emphasis on the development of selective and cost-effective BoNT therapeutics. Some of the experimental approaches being explored in this actively growing research area are summarized in Fig. 2.

Animal and human studies suggest that the presence of preformed, neutralizing antibodies in the serum to bind and eliminate toxin before it reaches target cells can prevent or reduce BoNT intoxication. Several different antitoxin products for human use to protect against BoNT have been developed. A "trivalent" (serotypes /A, /B, and /E) equine antitoxin product, as well as a monovalent BoNT/E antitoxin, are licensed by Aventis Pateur Canada (formerly Connaught Laboratories, Ltd.) and approved for use in the United States. Biomed of Warsaw, Poland also produces a trivalent BoNT/A/B/E anti-toxin. Additionally, an experimental, despeciated equine heptavalent (serotypes /A-/G) product was developed at USAMRIID and currently is administered under an IND protocol for limited use.

Several efforts have been undertaken to produce a human antibody-based therapeutic since the widespread clinical recognition of infant botulism in the late 1970s *(11)*. In 1981–1982, the US Army, the California Department of Health Services, and the

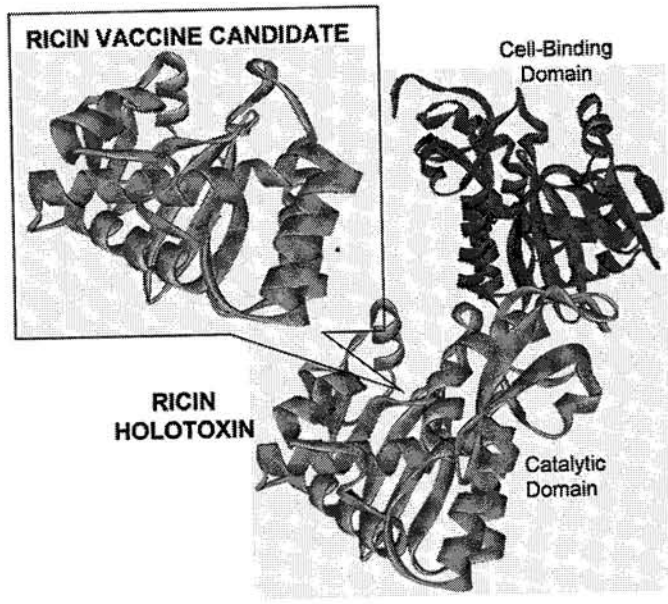

Fig. 4. Topology of recombinant ricin vaccine candidates with respect to the overall three-dimensional structure of the holotoxin. The figure shows a ribbon diagram of the ricin holotoxin structure solved by X-ray crystallography (PDB entry 2AAI). The holotoxin includes two subunits, RTA (light gray), and RTB (dark gray). The hypothetical structure of the domain (RTA1-198) used for recombinant vaccine candidates is projected from the toxin structure based on the known amino acid sequence.

University of Minnesota collaborated to produce a human botulism immune globulin (BIG) antiserum from different pools of plasma obtained from human donors who had been vaccinated previously with the PBT vaccine. Subsequently, a human antitoxin (BIG-IV) was developed and distributed for treating infant botulism under a United States Food and Drug Administration (FDA)-authorized IND protocol *(11)*. Intravenous administration of BIG significantly reduces the hospital stay of infants diagnosed with botulism, but it is not clear to what extent the success of BIG with infant botulism will also apply to treating patients exposed to BoNT by aerosol. Moreover, because of the obvious logistical barriers in production of antiserum in humans, the available BIG supplies are not expected to meet the mass casualty demands of a BW attack.

There are several ongoing biotechnological approaches aimed at expanding the availability of anti-toxins for human use. It is beyond the scope of this chapter to review all promising technologies underway, but we offer two examples currently being explored: the design and production of human recombinant monoclonal antibodies (MAbs) and the production of transgenic animals capable of producing human antibodies.

Preliminary studies showed that MAbs produced in rodents could neutralize large amounts of BoNT toxin, at least 10–100 times the BoNT toxin LD_{50} doses *(45,64)*. Subsequent work by Marks et al. generated phage antibody libraries from mice vaccinated with the Hc neutralizing epitope identified in earlier studies or from human volunteers previously vaccinated with PBT *(65,66)*. The technology permits recombinant expression of human antibodies for potential use as therapeutics. Rapidly evaluating a

relatively large number of unique MAbs from these libraries has opened up the possibility of achieving a multiplicative increase in toxin neutralization by combining high-affinity antibodies with nonoverlapping binding sites. Using this approach under optimal conditions, it has been possible to neutralize very large amounts of BoNT/A, thereby providing protection in animal models against greater than 10,000–100,000 times the toxin LD_{50} doses *(67)*. The medical product expected from this research will be well-defined "oligoclonal" mixtures of selected human MAbs specific for toxin serotypes.

An alternative approach to achieve large-scale production of high-affinity therapeutic antibodies for BoNT or other toxins is the use of human artificial chromosome vectors to introduce the entire, unrearranged sequences for human Ig light- and heavy-chain genes into livestock. Kuroiwa et al. demonstrated that a human artificial chromosome vector can be inserted into bovine fetal fibroblast cells, thereby allowing for the production of cloned cattle carrying the human antibody genes *(68)*. Such animals could be used to produce large quantities of human polyclonal antisera against BoNT or other toxins without the side effects and logistical burden inherent in the past clinical use of despeciated antisera.

3.5. *Small-Molecule Experimental Therapeutics*

There currently is no safe and effective small-molecule therapeutic for preventing or reversing BoNT intoxication. During the past several years, however, a more-detailed understanding of the complex steps involved in BoNT intoxication, including neuron binding, translocation, and catalysis, has opened up the possibility for rational development of therapeutic intervention at the molecular level.

Ongoing research has focused on BoNT active-site inhibitors, including peptide-based captopril derivatives and other classes of zinc metalloprotease inhibitors *(69–72)*. Most of the inhibitors reported to date are either nonselective or bind with affinity that is too low to be useful as a therapeutic. Iterative inhibitor design is improving the situation and recently a β-amino thiol inhibitor of BoNT B was reported with a *Ki* of 20 n*M (73,74)*. Technical challenges to this approach include the use of peptide derivatives as drugs, the difficulty in delivering drugs within the nervous system, and the likely need to develop specific inhibitors for each serotype of BoNT.

With the goal of arriving at therapeutics that will antagonize multiple serotypes of BoNT, small molecules that act indirectly to overcome the presynaptic blockade of neurotransmitter release also have been explored. Toosendanin, a triterpenoid derivative from the bark of *Melia toosendan*, has limited efficacy in antagonizing the effects of BoNT intoxication in cell-based systems, as well as in a preliminary nonhuman primate study *(75–77)*. The mechanism of action for Toosendanin remains unclear, although it appears to act as a complex presynaptic blocking agent that can alter the quantal release of ACh by modulating calcium channel activity *(78,79)*. However, toxicity is expected to limit its usefulness as a drug because Toosendanin itself blocks presynaptic ACh release under conditions close to those found to show efficacy against BoNT.

The potassium channel blocker, 3,4-diaminopyridine (3,4-DAP) also can antagonize the effects of BoNT in vivo, provided that the drug concentration is maintained at plasma concentrations of about 30 μ*M* during the entire clinical course of intoxication

(80,81). The results were comparable when 3,4-DAP was given 1, 2, 3, or 7 d after BoNT/A intoxication, but the compound was also essentially ineffective in antagonizing the paralytic actions of BoNT/B or BoNT/F. As with Toosendanin, one expects that the general toxicity of 3,4-DAP, coupled with the requirement of prolonged drug administration at relatively high levels, preclude routine therapeutic use.

Other experimental approaches being explored as future BoNT therapeutics include receptor antagonists as pretreatments that selectively block toxin receptor binding, as well as therapies that reverse intoxication by replacing target molecules destroyed by the toxin. An example of the latter is the replacement of SNARE proteins by gene therapy to rescue BoNT-intoxicated neurons *(82)*.

4. *STAPHYLOCOCCUS AUREUS* ENTEROTOXINS

Whereas BoNT achieves its potency by dampening an amplified extracellular signal of nerve cells via enzymatic catalysis, the *S. aureus* enterotoxins (SE) operate by inappropriately amplifying an extracellular signal of key immune cells. The SE belong to an extended family of stable 23–29 kD protein toxins that includes SE serotypes /A, /B, /Cn, /D, /E, and /H, and streptococcal pyrogenic exotoxins serotypes /A-/C; /F-/H, and /J, as well as toxic shock syndrome toxin (TSST-1) *(83)*. Based on their common ability to cause severe illness in animals by inducing a physiological overreaction of the host-immune response, these toxins have been categorized collectively as "superantigens" (SAgs); they are a major cause of human food poisoning and also contribute significantly to opportunistic bacterial infections in hospital patients (reviewed in refs. *84–88*).

The most important SAg in the context of BW is SE serotype /B (SE/B). SE/B is a two-domain, α–β-protein that contains discrete binding sites for the major histocompatibility complex (MHC) class II molecule and the Vβ regions of T-cell antigen receptors (TCRs) *(89–91)*. By binding to these two receptor molecules, and perhaps through other cell–surface interactions, SE/B is able to activate both antigen-presenting cells and a relatively large number of T lymphocytes to cause release of pyrogenic cytokines, chemokines, and other proinflammatory molecules (reviewed in ref. *92*).

The more common forms of SAg food poisoning can be managed with routine supportive care, but SE/B poses a formidable aerosol threat because of its high potency and stability. It is estimated that SE/B can produce human incapacitation and death at levels as low as 0.03 and 1.5 μg, respectively, by the aerosol route of exposure.[3] Primates are more sensitive than are many other animal species, perhaps partly because of higher affinity of the primate MHC class II receptors for SE/B. In a rhesus monkey model, exposure to lethal amounts of SE/B caused disabling emesis followed by a rapid drop in blood pressure, elevated temperature, skin rashes, toxic shock, multiple organ failure, and death (reviewed in ref. *93*).

4.1. *SE Toxoid Vaccines*

Almost 40 yr ago, it was shown that SE/B can be isolated from bacterial culture supernatants in highly purified form, and inactivated with neutral formaldehyde solu-

[3]These values are estimates for a 70-kg human based upon extrapolation of limited laboratory reports of accidental intoxication; the aerosol LD_{50} in primates is 20–30 μg/kg.

tions to produce an effective toxoid vaccine *(94)*. Anecdotal safety problems were reported during early animal studies of an SE/B toxoid vaccine, perhaps because there was not a standard methodology for making the toxoid *(95–97)*. Warren et al. systematically characterized different conditions of pH and formaldehyde concentration and suggested that conditions for crosslinking the toxin were critical for obtaining reproducible and immunogenic vaccine candidates; from these studies, an effective SE/B toxoid vaccine was later produced by incubating toxin with 1% formaldehyde at 37°C for 30 d at pH 7.5 *(98,99)*.

The SE/B toxoid combined with a suitable adjuvant or enhancer protected monkeys from an SE/B aerosol challenge of greater than 10 LD_{50} *(100–102)*. Complexes of the SE/B toxoid with selected components of the meningococcal outer membrane (so-called "SE/B toxoid proteosomes"), for example, induced protective immunity intranasally or intramuscularly, and protected 100% of monkeys challenged with SE/B aerosol *(102,103)*. Nevertheless, the requirement of active toxin production as starting material, the possibility of toxoid reversion to yield active SE/B toxin, as well as minor reactogenicity associated with formaldehyde-inactivated vaccines, has prompted research to develop improved SE/B vaccines.

4.2. *Engineered SE Vaccines*

Comparative structural and biochemical studies carried out during the 1990s focused on the development of nontoxic, recombinant immunogens capable of eliciting a protective immune response against multiple SAg toxins *(83,104)*. Ulrich et al. at USAMRIID attempted to inactivate SE by modifying three structural regions of the toxin that are involved in HLA-DR1 binding: a polar pocket created by three β-strand elements of the β-barrel domain of the toxin, a hydrophobic reverse turn, and a disulfide-bonded loop *(104)*.

The polar binding pocket binding region in SE/B comprises three key residues of the toxin, Glu67, Tyr89, and Tyr115, that are postulated to form favorable interactions with Lys39 of the HLA-DR1 α-subunit by ion-pairing and hydrogen bonding *(90,105,106)*. Removing the anion at Glu67 (Glu→Gln substitution) resulted in an approx 100-fold reduction in binding affinity; substituting either Tyr89 or Tyr115 with Ala also reduced binding by 100-fold *(104,105)*.

A second critical binding region between SE/B and the HLA-DR molecule involves a hydrophobic reverse turn region comprising amino acid residues 44–47 that connects β-strands 1 and 2 of SE/B *(105,107)*. The backbone atoms of these residues are positioned to participate in attractive electrostatic interactions with the HLA-DR1 α-subunit; there also appears to be favorable hydrophobic packing between the toxin and the receptor mediated by SE/B Leu45.

Replacing Leu45 with a large, polar residue (Leu→Arg substitution) reduced binding of the toxin to HLA-DR1 to below detectable limits *(105)*. The Leu45Arg substitution may alter the volume and polarity of this small hydrophobic pocket of SE/B sufficiently to disrupt its binding with HLA-DR1. Vaccinating mice with Leu45Arg or with site-specific mutants designed to disrupt backbone contacts within the reverse turn region (Gln43Pro and Phe44Pro), protected against a supralethal (approx 30 times LD_{50}) challenge with SE/B; the Leu45Arg mutant also induced a greater IgG2a and IgG2b immune response in vaccinated mice than did either of the Pro mutants *(108)*.

Finally, a disulfide bonded loop region of the SE/B structure was implicated in receptor binding by analogy with TSST results, and substitution within this region (Tyr94→Ala) reduced binding of SE/B with HLA-DR1 *(104)*.

By combining substitutions in each of these three structural regions of SE/B (Tyr89Ala, Leu45Arg, and Tyr94Ala) within a single immunogen, a recombinant vaccine candidate (rSE/Bv) was produced that lacks detectable SAg activity. An analogous recombinant immunogen subsequently was developed for SE type A (SE/A) by introducing comparable substitutions: Asp70Arg, Leu48Arg, and Tyr92Ala. Figure 3 depicts the relative positions of the altered side chains within the solved, three-dimensional X-ray crystal structure of the SE/A immunogen *(109)*.

The rSE/Bv was tested in rodent[4] and nonhuman primate model systems for safety and efficacy. The vaccine elicited high antibody titers, and vaccinated mice survived supralethal challenges with SE/B toxin. When administered at a 20-μg dose, three-dose schedule, using alum as adjuvant, rSE/Bv protected nonhuman primates against greater than 20 LD_{50} of SE/B toxin. Moreover, in contrast with natural toxin, rSE/Bv showed no evidence of toxic SAg activity. In ex vivo assays conducted with human immune cells, rSE/Bv did not bind human MHC class II receptors; did not stimulate cytokine release; and did not elicit nonspecific T-cell cell mitosis. A suitable process has been developed to produce rSE/Bv in a high-level *E. coli* expression system under conditions compatible with current Good Manufacturing Practices *(111)*.

Additional structural and mutation studies have been undertaken to produce analogous vaccine candidates for protection against other SAgs *(83,105,112–114)*. Suggestive evidence has been presented that vaccination with SE/B and SE/A vaccine candidates may offer some protection against other SE toxin serotypes, raising the possibility of a single vaccine that can offer protection against multiple SAgs.

4.3. Experimental Therapeutics

There is no approved therapeutic for reversing the effects of SE intoxication; treatment is aimed at reducing the incapacitating symptoms, maintaining adequate hydration, and preventing or managing the clinical sequelae of systemic shock.

Several experimental approaches are underway to evaluate potential therapeutic approaches to SE. These include suppressing abnormal T-cell activation by preventing or disrupting abnormal TCR–MHC interactions; mitigating downstream cytokine or chemokine release caused by activated lymphocytes and macrophages after SAg exposure; and blocking the costimulatory molecules involved in activation or other effector functions of T cells *(115–117)*.

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[4]Unlike the MHC receptors of humans and other primates, the α-chain of mouse MHC class II lacks key amino acid residues involved in high affinity toxin binding. For this reason, mouse models of SE/B exposure require addition of a potentiating molecule, such as lipopolysaccharide preparations from Gram-negative bacteria, along with the toxin to model the severe incapacitation observed in primates exposed to SE/B alone. Recently, it has become possible to test SE/B vaccine candidates in transgenic mice expressing human leukocyte antigen (HLA)-DR3 and human CD4 molecules, in the absence of murine major histocompatibility complex (MHC) class II molecules (see ref. *110*. DaSilva, L., et al. (2002) Humanlike immune response of human leukocyte antigen-DR3 transgenic mice to staphylococcal enterotoxins: a novel model for superantigen vaccines. *J. Infect. Dis.* **185(12),** 1754–1760.) This animal model eliminates the need for toxin potentiation with lipopolysaccharide.

5. RICIN TOXIN

Ricin is a disulfide-bonded, heterodimeric toxin from the seeds of *Ricinus communis* (castor bean plant) that has been recognized as a potential toxin weapon since World War I *(118)*. Although much less lethal by weight than BoNT or SE *(119–121)*, ricin is nevertheless a potent BW agent because sublethal doses cause incapacitating pulmonary damage and because the toxin is widely available; the castor bean plant is cultivated worldwide for several beneficial applications, and the toxin is easily extracted from common byproducts of the seeds *(122,123)*.

Entry of ricin into target cells is greatly enhanced by the ricin B-chain (RTB); RTB is a galactose-specific lectin that binds receptors on the surface of target cells, thereby promoting endocytosis and trafficking to the trans Golgi. The ricin A-chain (RTA) is a multidomain *N*-glycosidase that depurinates a specific adenosine of the essential 60S ribosomal RNA (rRNA) subunit (reviewed in ref. *124*). Once eukaryotic rRNA has been damaged in this way, the target cell cannot synthesize new protein and inevitably will die. Ricin is representative of a diverse class of "ribosome inactivating proteins" (RIPs) that includes the plant toxins abrin, modeccin and viscumin, as well as several potent bacterial toxins *(125,126)*.

Human poisoning by ricin aerosol exposure is not documented, but based on extrapolation from accidental, human sublethal exposures, the signs and symptoms are likely to include high fever, dyspnea, and coughing that is delayed for 4–8 h after exposure *(123,127,128)*. In nonhuman primates, aerosolized ricin causes a dose-dependent set of signs that is delayed from 8 to 24 h; anorexia and lethargy are frequently observed. In one study of rhesus monkeys exposed to approx 20–40 μg/kg of ricin aerosol, death occurred by acute respiratory distress about 36–48 h after exposure; necropsy revealed fibrinopurulent pneumonia, acute inflammation of trachea and airways, and massive pulmonary alveolar flooding *(129)*.

5.1. Ricin Toxoid Vaccines

Toxoid vaccine prepared from formalin-inactivated ricin holotoxin was developed during World War II and shown to enhance survival significantly in animals exposed to ricin *(118)*. An improved ricin toxoid vaccine based on denatured toxin adsorbed to Alhydrogel adjuvant was developed at USAMRIID in the 1990s and shown to be effective at protecting rhesus monkeys against ricin toxin aerosol exposures. All vaccinated monkeys survived a supralethal ricin aerosol challenge; however, as with earlier studies, vaccination did not protect completely against short-term (up to 14 d postexposure) bronchiolar and interstitial pulmonary inflammation. The general failure of toxoid vaccines to protect the respiratory tract of exposed animals from the cytotoxic effects of ricin underscores the need to develop effective recombinant vaccines and alternative vaccine-delivery systems that can elicit an enhanced mucosal immune response *(121,130)*.

5.2. Deglycosylated Ricin A-Chain Vaccine

RTA conjugated with tumor-specific antibodies has been used clinically as medicotoxin to target and kill tumors in animals and humans *(131,132)*. Supporting studies with RTA-antibody conjugates contributed to the development of a recombinant ricin vaccine because they demonstrated unequivocally that RTA is much less

toxic than is the whole toxin when administered parenterally to animals in the absence of the RTB *(133,134)*.

During the early 1990s, researchers at USAMRIID demonstrated that purified RTA can act as an effective immunogen in animals to elicit antibodies that neutralize whole ricin toxin *(135,136)*. A suitable lot (meeting current Good Manufacturing Practices) of chemically degylosylated (dg) RTA subsequently was produced from the natural toxin and shown to protect against supralethal ricin aerosol challenges in two animal models.[5] However, technical limitations were raised regarding the use of RTA or dgRTA as a human vaccine candidate; both immunogens retain residual *N*-glycosidase activity and show significant aggregation during expression, purification, or upon prolonged storage in solution.

Recombinant vaccine candidates with active-site specific substitutions designed to reduce the *N*-glycosidase activity of RTA without disrupting the antigenic properties of the molecule have been proposed as vaccine candidates *(135,137–139)*. Some of these recombinant candidates also have been altered to remove a putative "vascular leak peptide" sequence reported to contribute to the toxicity observed with very high levels of RTA used in immunotoxin chemotherapy studies *(132,139)*. Active-site substitutions in RTA essentially eliminate the problem of residual toxic activity but do not address the important manufacturing problem of RTA instability and aggregation.

5.3. *Engineered Ricin Vaccines*

Olson recognized that the tendency of subunit-based RTA vaccines to self-aggregate under physiological conditions was related to hydrophobic domains exposed by the absence of the natural RTB subunit. Starting from a theoretical analysis of the functional architecture of the toxin compared with related single-chain RIPs *(140–142)*, it was hypothesized that reducing the hydrophobic surface of RTA by large-scale deletions might result in a better structural platform for presenting the neutralizing epitope than that of the parent molecule.

Along with a reduced hydrophobic surface, recombinant vaccine candidates were required to retain the surface loop that is believed to serve as a neutralizing immunological epitope for ricin toxin (RTA residues 97–106; ref. *137*). Candidates also were required to lack key amino acid residues of the RNA binding site that are essential for toxic *N*-glycosidase activity. From experimental trials with a range of recombinant RTA candidates, we found that immunogens based approximately on the *N*-terminal domain of RTA (residues 1–198) best satisfied the design criteria (Fig. 4).

Under physiological conditions, polypeptides based on RTA1-198 remain folded as judged by circular dichroism and infrared spectroscopy, are more stable thermodynamically than is RTA, and exhibit dynamic light scattering indicating monodisperse monomers without significant aggregation. Moreover, the single-domain immunogens show no detectable toxin activity and protect mice against supralethal exposure to ricin toxin by injection or by aerosol. In this case, protein engineering based partly on a functional analysis of protein domains has yielded ricin vaccine candidates that are superior to traditional approaches, including inactivated holotoxin or toxin subunit vaccines containing simple active-site mutations.

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[5]Unpublished observations of Dr. R.W. Wannemacher, USAMRIID, Fort Detrick, MD 21010.

delivered by genetically engineered bacteria or viruses in unnatural amounts or combinations to the wrong organ, target cell, or compartment; examples of such adventitious "toxins" may include polypeptide hormones, growth factors, and cytokines or other immune system modulators *(146)*.

It is clear that if toxins are considered in the context of genetically engineered microbes, then it is not possible at present to delimit the complete set of potential protein toxins. However, if we assume that engineered protein toxin weapons initially will likely employ or modify existing natural toxin scaffolds or functions, then the problem becomes more tractable. Such future threats might involve, for example, the conversion of structural neighbors of known toxins into closely related toxins; novel chimeras comprising known toxin subunits or domains; or protein engineering and co-/post-translational modifications employed to defeat natural immunity, approved vaccines, or detection systems.

Although the rational design of protein toxins remains largely impractical at present, biotechnology and understanding of protein structure have started to test this limit in two specific areas: (a) subunit combinations to create toxin chimeras, and (b) building upon common structural scaffolds to transfer function among polypeptide toxins. Growing interest in these areas is driven by potential benefits of medicotoxins, as well as the power of using toxins in basic biomedical research to selectively perturb biological systems.

6.1. Protein Toxin Chimeras

Many protein toxins operate by combining relatively diverse functions, such as binding receptors on target cells, promoting toxin internalization (membrane translocation), intracellular trafficking to target compartments, and subsequently exerting a toxic intracellular effect such as hydrolysis of an essential cellular component. The structure of natural toxins often exhibits a corresponding multiplicity, with functions partitioned among different polypeptide subunits or domains. During the past several years, it has become possible to attribute specific functions to toxin parts and, using protein engineering, to produce synthetic toxin chimeras composed of unnatural combinations of binding and catalytic subunits.

Toxin chimeras have been employed clinically to target and kill unwanted cell types or tumors. This application hitches the most deadly bacterial toxin subunits, often the catalytic domain of a multichain RIP toxin, to a binding subunit or antibody (so-called "immunotoxin") that targets a particular receptor. For example, the active subunit of diphtheria toxin has been conjugated with an epidermal growth factor-like domain to target cells expressing specific receptors *(147,148)*. Similarly, the neuron-binding domain of one toxin, such as TeNT or BoNT Hc, has been combined with the diphtheria toxin RIP subunit *(149)*. Neurons also have been targeted by chimeras of diphtheria toxin RIP subunit and modified substance P; the result is a directed protoxin that is activated by a specific posttranslational modification *(150)*.

It has been shown that there is a degree of permissiveness in the types of catalytic or functional subunits that can be delivered into mammalian cells by toxin binding and translocation processes. For example, the catalytic domains from two of the most deadly bacterial toxins known, TeNT and Shiga toxin, have been combined with the anthrax toxin ensemble to produce cytotoxins that will target a broader class of mammalian cell

types *(151,152)*. Likewise, bacterial toxin translocation systems, for example, the pore-forming toxin streptolysin-*O*, are being developed as a means of intracellular delivery of relatively large (approx 100 kD) unrelated toxin subunits *(153)*.

Synthetic toxin chimeras are of interest in BW defense because, although they are expected to be less potent than parent molecules generally, the chimeras may result in confusing medical signs and symptoms. Chimeras also pose a challenging dilemma for medical diagnostics of BW casualties by reacting with detection systems for one toxin, while carrying the biological functionality of another.

6.2. *Modification of Natural Toxin Scaffolds*

Protein engineers currently lack understanding sufficient to permit *de novo* design and production of new toxins that are significantly more potent than the parent molecules. However, it has become increasingly feasible to engineer controlled modifications into existing protein toxin structures. The widespread appearance of diverse protein and polypeptide toxins in animal venoms has led to the manipulation of certain stable, natural protein scaffolds for the design or transfer of toxic function.

One permissive toxin scaffold is the "three-finger" fold employed by a number of single polypeptide chain animal toxins *(154)*. This toxin family fold is based on what is primarily a β-sheet protein core that is greatly stabilized by disulfide bonds coupled with highly variable surface loops that tolerate significant structural changes because of the stability of the protein core. Menez et al. applied structural and molecular biology to alter venom toxin binding specificity in the design and synthesis of a hybrid toxin that retains more than 50% identity to one toxin (toxin-α), while binding the natural target molecule, acetylcholinesterase, of a second toxin, fasciculin-II (FASII), with high affinity *(155)*. A model of residues essential for binding of FASII with acetylcholinesterase was proposed based on primary sequence and structural homologies among a large number of three-finger snake toxins, as well as the solved three-dimensional structure of the FASII-acetylcholinesterase complex *(156)*. Although the work is remarkable for demonstrating the transfer of function from one toxin (FASII) onto the sequence of another, the hybrid toxin is much less potent than FASII, underscoring the limitations of understanding protein–protein interactions from structural studies alone.

Another permissive protein toxin scaffold may be the disulfide-stabilized α–β-fold employed in large families of scorpion venoms (reviewed in ref. *157*). Zilberberg et al. applied site-directed mutagenesis to modify the binding specificity of a scorpion neurotoxin (Lqh α IT) *(158)*. Additionally, the potency of one long-chain scorpion toxin (BotIX) could be enhanced by the transferring select residues from a scorpion alpha toxin (Lqh α IT) *(159)*.

Small, structural mimics of natural protein toxins are also a concern for the future. The increasingly fine detail available for how polypeptide toxins bind to critical cell receptors or ion channels may result in the development of toxic, low-molecular-weight oligopeptides or peptide mimetics. Venoms from *Conus* hunting snails provide a natural example of how relatively small polypeptides can produce a large repertoire of stable, diverse and functionally synergistic toxins ("conotoxins" reviewed in ref. *160*). Peptide mimetics that carry some of the toxicity of the holotoxin, but with a much lower molecular weight and greater stability, may effectively enhance the potency of known toxins.

7. CONCLUSION

During the past decade, effective vaccine candidates for several of the most dangerous toxin weapons, including BoNT, SE/B, and ricin, have been designed, produced, or improved by protein engineering. Vaccination remains the foundation of effective medical protection against macromolecular threats, but it is clearly impractical and, in some scenarios, unethical to vaccinate against all possible threat molecules. Additionally, there are military operational requirements for which BW vaccination regimens that require weeks to months may be too slow or inflexible. Effective toxin therapies, such as engineered human antitoxins or small-molecule antagonists, are needed as adjuncts or replacements for vaccination.

Future directions include reducing the size and/or increasing the binding stoichiometry of antitoxin molecules. Protein engineering of catalytic scavengers has shown increasing promise as a potential medical countermeasure for low-molecular-weight toxins *(161,162)*, and it may become possible to develop catalysts that combine the binding specificity of antitoxins with protease activity to selectively catalyze the hydrolysis or inactivation of protein toxins. Design or development of irreversible protease inhibitors as prophylactic antitoxins also merits increased emphasis.

This review intentionally emphasizes how protein engineering can be applied in medical research to effectively shorten the timescale of evolving natural defenses against toxins. This approach holds out tremendous promise for protection against many known chemical and BW agents, provided the toxin does not change structure appreciably during the lifetime of the vaccine or therapy. A darker, competing view holds that biotechnology, combined with an open scientific literature, may have the same powerful accelerating effect on emergence or creation of novel threat agents to confound our engineered vaccines or, alternatively, to short-circuit natural immune processes.

A key resource for devising better medical protection remains analyzed, high-resolution structural biology data. Existing bioinformatics, computational chemistry, and structural biology tools for large-scale, comparative analysis of solved protein structures, and mechanisms can be applied to protein toxins *(163–165)*. Knowledge of the set of structural "building blocks" for natural protein toxins will emerge and may serve to improve medical products for simultaneous neutralization of multiple toxin weapons. We must remain cognizant, however, that the growing markets for beneficial medicotoxins against a range of human illnesses will continue to drive protein engineering of toxin chimeras, as well as new means to develop tolerance or defeat immunity to extend the clinically useful lifetime of toxin-based drugs. Consequently, researchers with a commitment to medical defense against BW agents should expect increasingly to wield, as well as to encounter, the double-edged sword of protein engineering.

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REFERENCES

1. Franz, D. R. (1997) Defense against toxin weapons, in *Medical Aspects of Chemical and Biological Warfare* (Sidell, F. R., Takafuji, E. T., and Franz, D. R., eds.), Office of the Surgeon General, Department of the Army, United States of America: Washington, D.C. pp. 603–620.
2. Gill, D. M. (1982) Bacterial toxins: a table of lethal amounts. *Microbiol. Rev.* **46(1),** 86–94.
3. Hatheway, C. L. (1990) Toxigenic clostridia. *Clin. Microbiol. Rev.* **3(1),** 66–98.
4. Paddle, B. M. (2003) Therapy and prophylaxis of inhaled biological toxins. *J. Appl. Toxicol.* **23(3),** 139–170.
5. Dack, G. M. (1956) *Food Poisoning*. Third ed. The University of Chicago Press, Chicago, p. 251.
6. Morgan, J. C. and Bleck, T. P. (2002) Clinical aspects of tetanus, in *Scientific and Therapeutic Aspects of Botulinum Toxin.* (Brin, M. F., Jankovic, J., and Hallett, M., eds.), Lippincott Williams & Wilkins, Philadelphia, PA, pp. 151–164.
7. Newman, M. J. and Powell, M. F. (1995) Immunological and formulation design considerations for subunit vaccines, in *Vaccin Design: The Subunit and Adjuvant Approach.* (Powell, M. F. and Newman, M. J., eds.), Plenum Press, New York, pp. 1–42.
8. Mikszta, J. A., et al. (2002) Improved genetic immunization via micromechanical disruption of skin-barrier function and targeted epidermal delivery. *Nat. Med.* **8(4),** 415–419.
9. Aoki, K. R. (2002) Physiology and pharmacology of therapeutic botulinum neurotoxins. *Curr. Prob. Dermatol.* **30,** 107–116.
10. Jankovic, J. (2002) Botulinum toxin: clinical implications of antigenicity and immunoresistance, in *Scientific and Therapeutic Aspects of Botulinum Toxin.* (Brin, M. F., Jankovic, J., and Hallett, M., eds.), Lippincott Williams & Wilkins, Philadelphia, PA, 409–415.
11. Arnon, S. S., et al. (2001) Botulinum toxin as a biological weapon: medical and public health management. *JAMA* **285(8),** 1059–1070.
12. Simpson, L. L. (1981) The origin, structure, and pharmacological activity of botulinum toxin. *Pharmacol. Rev.* **33(3),** 155–188.
13. Shapiro, R. L., Hatheway, C., and Swerdlow, D. L. (1998) Botulism in the United States: a clinical and epidemiologic review. *Ann. Intern. Med.* **129(3),** 221–228.
14. Franz, D. R., Parrott, C. D., and Takafuji, E. T. (1997) The U. S. Biological Warfare and Biological Defense Programs, in *Medical Aspects of Chemical and Biological Warfare* (Sidell, F. R., Takafuji, E. T., and Franz, D. R., eds.), Office of the Surgeon General, Department of the Army, United States of America, Washington, D.C., pp. 425–436.
15. Umland, T. C., et al. (1997) Structure of the receptor binding fragment HC of tetanus neurotoxin. *Nat. Struct. Biol.* **4(10),** 788–792.
16. Lacy, D. B., et al. (1998) Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nat. Struct. Biol.* **5(10),** 898–902.
17. Lacy, D. B. and Stevens, R. C. (1999) Sequence homology and structural analysis of the clostridial neurotoxins. *J. Mol. Biol.* **291(5),** 1091–1104.
18. Eswaramoorthy, S., Kumaran, D., and Swaminathan, S. (2001) Crystallographic evidence for doxorubicin binding to the receptor-binding site in *Clostridium botulinum* neurotoxin B. *Acta Crystallogr. D Biol. Crystallogr.* **57(Pt 11),** 1743–1746.
19. Montecucco, C. (1986) How do tetanus and botulinum toxins bind to neuronal membranes? *Trends Biochem. Sci.* **11,** 314–317.
20. Fujinaga, Y., et al. (1997) The haemagglutinin of *Clostridium botulinum* type C progenitor toxin plays an essential role in binding of toxin to the epithelial cells of guinea pig small intestine, leading to the efficient absorption of the toxin. *Microbiology* **143(Pt 12),** 3841–3847.

21. Koriazova, L. K. and Montal, M. (2003) Translocation of botulinum neurotoxin light chain protease through the heavy chain channel. *Nat. Struct. Biol.* **10(1),** 13–18.
22. Sheridan, R. E. (1998) Gating and permeability of ion channels produced by botulinum toxin types A and E in PC12 cell membranes. *Toxicon* **36(5),** 703–717.
23. Simpson, L. L. (1986) Molecular pharmacology of botulinum toxin and tetanus toxin. *Annu. Rev. Pharmacol. Toxicol.* **26,** 427–453.
24. Poulain, B., et al. (1991) Heterologous combinations of heavy and light chains from botulinum neurotoxin A and tetanus toxin inhibit neurotransmitter release in Aplysia. *J. Biol. Chem.* **266(15),** 9580–9585.
25. Hanson, P. I., Heuser, J. E., and Jahn, R. (1997) Neurotransmitter release—four years of SNARE complexes. *Curr. Opin. Neurobiol.* **7(3),** 310–315.
26. Brunger, A. T. (2001) Structure of proteins involved in synaptic vesicle fusion in neurons. *Annu. Rev. Biophys. Biomol. Struct.* **30,** 157–171.
27. Rizo, J. (2003) SNARE function revisited. *Nat. Struct. Biol.* **10(6),** 417–419.
28. Reames, H. R., et al. (1947) Studies on botulinum toxoids, types A and B III. Immunization in man. *J. Immunol.* **55,** 309–324.
29. Sterne, M. and Wentzel, L. M. (1950) A new method for the large-scale production of high-titre botulinum formol-toxoid types C and D. *J. Immunol.* **65,** 175–183.
30. Fiock, M. A., Cardella, M. A., and Gearinger, N. F. (1963) Studies of immunities to toxins of Clostridium botulinum. IX. Immunologic response of man to purified pentavalent ABCDE botulinum toxoid. *J. Immunol.* **90,** 697–702.
31. Cardella, M. A. (1964) Botulinum toxoids, in *Botulism, Proceedings of a Symposium.* U. S. Public Health Service Publication No. 999-FP-1. (Lewis, Jr., K. H. a. C., ed.), Public Health Service, Cincinnati, OH, pp. 113–130.
32. Anderson, J. H. and Lewis, G. E. (1981) Clinical evaluation of botulinum toxoids, in *Biomedical Aspects of Botulism.* (Lewis, G. E., ed.), Academic Press, New York, pp. 233–246.
33. Oguma, K., Fujinaga, Y., and Inoue, K. (1995) Structure and function of *Clostridium botulinum* toxins. *Microbiol. Immunol.* **39(3),** 161–168.
34. Siegel, L. S. (1988) Human immune response to botulinum pentavalent (ABCDE) toxoid determined by a neutralization test and by an enzyme-linked immunosorbent assay. *J. Clin. Microbiol.* **26(11),** 2351–2356.
35. Hatheway, C. (1976) Toxoid of *Clostridium botulinum* type F: purification and immunogenicity studies. *Appl. Environ. Microbiol.* **31,** 234–242.
36. Torii, Y., et al. (2002) Production and immunogenic efficacy of botulinum tetravalent (A, B, E, F) toxoid. *Vaccine* **20(19-20),** 2556–2561.
37. Byrne, M. P. and Smith, L. A. (2000) Development of vaccines for prevention of botulism. *Biochimie* **82(9-10),** 955–966.
38. Fairweather, N. F., Lyness, V. A., and Maskell, D. J. (1987) Immunization of mice against tetanus with fragments of tetanus toxin synthesized in *Escherichia coli. Infect. Immun.* **55(11),** 2541–2545.
39. Clayton, M. A., et al. (1995) Protective vaccination with a recombinant fragment of *Clostridium botulinum* neurotoxin serotype A expressed from a synthetic gene in *Escherichia coli. Infect. Immun.* **63(7),** 2738–2742.
40. LaPenotiere, H. F., Clayton, M. A., and Middlebrook, J. L. (1995) Expression of a large, nontoxic fragment of botulinum neurotoxin serotype A and its use as an immunogen. *Toxicon* **33(10),** 1383–1386.
41. Simpson, L. L. (1984) Fragment C of tetanus toxin antagonizes the neuromuscular blocking properties of native tetanus toxin. *J. Pharmacol. Exp. Ther.* **228(3),** 600–604.
42. Simpson, L. L. (1984) Botulinum toxin and tetanus toxin recognize similar membrane determinants. *Brain Res.* **305(1),** 177–180.

43. Helting, T. B. and Nau, H. H. (1984) Analysis of the immune response to papain digestion products of tetanus toxin. *Acta Pathol. Microbiol. Immunol. Scand. (C)* **92(1),** 59–63.
44. Thompson, D. E., et al. (1990) The complete amino acid sequence of the *Clostridium botulinum* type A neurotoxin, deduced by nucleotide sequence analysis of the encoding gene. *Eur. J. Biochem.* **189(1),** 73–81.
45. Dertzbaugh, M. T. and West, M. W. (1996) Mapping of protective and cross-reactive domains of the type A neurotoxin of *Clostridium botulinum*. *Vaccine* **14(16),** 1538–1544.
46. Middlebrook, J. L. (1995) Protection strategies against botulinum toxin. *Adv. Exp. Med. Biol.* **383,** 93–98.
47. Potter, K. J., et al. (1998) Production and purification of the heavy-chain fragment C of botulinum neurotoxin, serotype B, expressed in the methylotrophic yeast *Pichia pastoris*. *Protein Expr. Purif.* **13(3),** 357–365.
48. Byrne, M. P., et al. (1998) Purification, potency, and efficacy of the botulinum neurotoxin type A binding domain from *Pichia pastoris* as a recombinant vaccine candidate. *Infect. Immun.* **66(10),** 4817–4822.
49. Byrne, M. P., et al. (2000) Fermentation, purification, and efficacy of a recombinant vaccine candidate against botulinum neurotoxin type F from *Pichia pastoris*. *Protein Expr. Purif.* **18(3),** 327–337.
50. Potter, K. J., et al. (2000) Production and purification of the heavy chain fragment C of botulinum neurotoxin, serotype A, expressed in the methylotrophic yeast *Pichia pastoris*. *Protein Expr. Purif.* **19(3),** 393–402.
51. Woodward, L. A., et al. (2003) Expression of HC subunits from Clostridium botulinum types C and D and their evaluation as candidate vaccine antigens in mice. *Infect. Immun.* **71(5),** 2941–2944.
52. Bouvier, A., et al. (2003) Identifying and modulating disulfide formation in the biopharmaceutical production of a recombinant protein vaccine candidate. *J. Biotechnol.* **103(3),** 257–271.
53. Atassi, M. Z. (2002) Immune recognition and cross-reactivity of botulinum neurotoxins, in *Scientific and Therapeutic Aspects of Botulinum Toxin.* (Brin, M. F., Jankovic, J., and Hallett, M., eds.), Lippincott, Williams & Wilkins, Philadelphia, PA, pp. 385–408.
54. Swaminathan, S. and Eswaramoorthy, S. (2000) Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. *Nat. Struct. Biol.* **7(8),** 693–699.
55. Chaddock, J. A., et al. (2002) Expression and purification of catalytically active, non-toxic endopeptidase derivatives of *Clostridium botulinum* toxin type A. *Protein Expr. Purif.* **25(2),** 219–228.
56. Lee, J. S., et al. (2001) Candidate vaccine against botulinum neurotoxin serotype A derived from a Venezuelan equine encephalitis virus vector system. *Infect. Immun.* **69(9),** 5709–5715.
57. Park, J. B. and Simpson, L. L. (2003) Inhalational poisoning by botulinum toxin and inhalation vaccination with its heavy-chain component. *Infect. Immun.* **71(3),** 1147–1154.
58. Bennett, A. M., Perkins, S. D., and Holley, J. L. (2003) DNA vaccination protects against botulinum neurotoxin type F. *Vaccine* ***21(23),*** 3110–3117.
59. Foynes, S., et al. (2003) Vaccination against type F botulinum toxin using attenuated *Salmonella enterica* var Typhimurium strains expressing the BoNT/F H(C) fragment. *Vaccine* **21(11-12),** 1052–1059.
60. Kiyatkin, N., Maksymowych, A. B., and Simpson, L. L. (1997) Induction of an immune response by oral administration of recombinant botulinum toxin. *Infect. Immun.* **65(11),** 4586–4591.
61. Simpson, L. L., Maksymowych, A. B., and Kiyatkin, N. (1999) Botulinum toxin as a carrier for oral vaccines. *Cell Mol. Life Sci.* **56(1-2),** 47–61.
62. Zdanovsky, A. G. and Zdanovskaia, M. V. (2000) Simple and efficient method for heterologous expression of clostridial proteins. *Appl. Environ. Microbiol.* **66(8),** 3166–3173.

63. Bradshaw, M., Goodnough, M. C., and Johnson, E. A. (1998) Conjugative transfer of the *Escherichia coli-Clostridium perfringens* shuttle vector pJIR1457 to *Clostridium botulinum* type A strains. *Plasmid* **40(3),** 233–237.
64. Pless, D. D., et al. (2001) High-affinity, protective antibodies to the binding domain of botulinum neurotoxin type A. *Infect. Immun.* **69(1),** 570–574.
65. Amersdorfer, P., et al. (1997) Molecular characterization of murine humoral immune response to botulinum neurotoxin type A binding domain as assessed by using phage antibody libraries. *Infect. Immun.* **65(9),** 3743–3752.
66. Amersdorfer, P., et al. (2002) Genetic and immunological comparison of anti-botulinum type A antibodies from immune and non-immune human phage libraries. *Vaccine* **20(11-12),** 1640–1648.
67. Nowakowski, A., et al. (2002) Potent neutralization of botulinum neurotoxin by recombinant oligoclonal antibody. *Proc. Natl. Acad. Sci. USA* **99(17),** 11,346–11,350.
68. Kuroiwa, Y., et al. (2002) Cloned transchromosomic calves producing human immunoglobulin. *Nat. Biotechnol.* **20(9),** 889–894.
69. Adler, M., et al. (1994) Evaluation of captopril and other potential therapeutic compounds in antagonizing botulinum toxin-induced muscle paralysis, in *Therapy with Botulinum Toxin* (Jankovic, J. and Hallett, M., eds.), Marcel Dekker, New York, pp. 63–70.
70. Adler, M., et al. (1998) Efficacy of a novel metalloprotease inhibitor on botulinum neurotoxin B activity. *FEBS Lett.* **429(3),** 234–238.
71. Schmidt, J. J., Stafford, R. G., and Millard, C. B. (2001) High-throughput assays for botulinum neurotoxin proteolytic activity: serotypes A, B, D, and F. *Anal. Biochem.* **296(1),** 130–137.
72. Schmidt, J. J. and Stafford, R. G. (2002) A high-affinity competitive inhibitor of type A botulinum neurotoxin protease activity. *FEBS Lett.* **532(3),** 423–426.
73. Anne, C., et al. (2003) Development of potent inhibitors of botulinum neurotoxin type B. *J. Med. Chem.* **46(22),** 4648–4656.
74. Anne, C., et al. (2003) Thio-derived disulfides as potent inhibitors of botulinum neurotoxin type B: implications for zinc interaction. *Bioorg. Med. Chem.* **11(21),** 4655–4660.
75. Zou, J., et al. (1985) The effect of toosendanin on monkey botulism. *J. Tradit. Chin. Med.* **5(1),** 29, 30.
76. Wang, Z. F. and Shi, Y. L. (2001) Toosendanin-induced inhibition of small-conductance calcium-activated potassium channels in CA1 pyramidal neurons of rat hippocampus. *Neurosci. Lett.* **303(1),** 13–16.
77. Xu, Y. and Shi, Y. (1993) Action of toosendanin on the membrane current of mouse motor nerve terminals. *Brain Res.* **631(1),** 46–50.
78. Shih, Y. L. (1986) Abolishment of non-quantal release of acetylcholine from the mouse phrenic nerve endings by toosendanin. *Jpn. J. Physiol.* **36(3),** 601–605.
79. Ding, J., Xu, T. H., and Shi, Y. L. (2001) Different effects of toosendanin on perineurially recorded Ca(2+) currents in mouse and frog motor nerve terminals. *Neurosci. Res.* **41(3),** 243–249.
80. Adler, M., et al. (1996) Effect of 3,4-diaminopyridine on rat extensor digitorum longus muscle paralyzed by local injection of botulinum neurotoxin. *Toxicon* **34(2),** 237–249.
81. Adler, M., Capacio, B., and Deshpande, S. S. (2000) Antagonism of botulinum toxin A-mediated muscle paralysis by 3, 4-diaminopyridine delivered via osmotic minipumps. *Toxicon* **38(10),** 1381–1388.
82. O'Sullivan, G. A., et al. (1999) Rescue of exocytosis in botulinum toxin A-poisoned chromaffin cells by expression of cleavage-resistant SNAP-25. Identification of the minimal essential C-terminal residues. *J. Biol. Chem.* **274(52),** 36,897–36,904.
83. Ulrich, R. G., Bavari, S., and Olson, M. A. (1995) Bacterial superantigens in human disease: structure, function and diversity. *Trends Microbiol.* **3(12),** 463–468.
84. Spero, L., Johnson-Winegar, A., and Schmidt, J. J. (1988) Enterotoxins of Staphylococci, in *Bacterial Toxins: Handbook of Natural Toxins* (Hardegree, M. C. and Tu, A. T. eds.), Marcel Dekker, New York, pp. 131–163.

85. Bohach, G. A., et al. (1996) The staphylococcal and streptococcal pyrogenic toxin family. *Adv. Exp. Med. Biol.* **391,** 131–154.
86. Sundberg, E. J., Li, Y., and Mariuzza, R. A. (2002) So many ways of getting in the way: diversity in the molecular architecture of superantigen-dependent T-cell signaling complexes. *Curr. Opin. Immunol.* **14(1),** 36–44.
87. Dinges, M. M., Orwin, P. M., and Schlievert, P. M. (2000) Exotoxins of *Staphylococcus aureus. Clin. Microbiol. Rev.* **13(1),** 16–34, table of contents.
88. Ulrich, R. G. (2000) Evolving superantigens of *Staphylococcus aureus. FEMS Immunol. Med. Microbiol.* **27(1),** 1–7.
89. Swaminathan, S., et al. (1988) Crystallization and preliminary X-ray study of staphylococcal enterotoxin B. *J. Mol. Biol.* **199(2),** 397.
90. Swaminathan, S., et al. (1992) Crystal structure of staphylococcal enterotoxin B, a superantigen. *Nature* **359(6398),** 801–806.
91. Swaminathan, S., et al. (1995) Residues defining V beta specificity in staphylococcal enterotoxins. *Nat. Struct. Biol.* **2(8),** 680–686.
92. Krakauer, T. (1999) Immune response to staphylococcal superantigens. *Immunol. Res.* **20,** 163–173.
93. Ulrich, R. G., et al. (1997) Staphylococcal enterotoxin B and related pyrogenic toxins, in *Medical Aspects of Chemical and Biological Warfare* (Sidell, F. R., Takafuji, E. T., and Franz, D. R., eds.), Office of the Surgeon General, Department of the Army, United States of America, Washington, D.C., pp. 621–630.
94. Schantz, E. J., et al. (1965) Purification of staphylococcal enterotoxin B. *Biochemistry* **4,** 1011–1016.
95. McGann, V. G. (1969) Evaluation of immunity against staphylococcal enterotoxin B. Commission on Epidemiological Survey, Annual Report to the Armed Forces.
96. McGann, V. G., et al. (1970) Immunological studies with microbial toxins. Research and Techology Work Unit Summary. Annual Progress Report. U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD.
97. Denniston, J. C., et al. (1970) Hypersensitivity reaction to staphylococcal enterotoxin B. Commission on Eppidemiological Survey. Annual Report to the Armed Forces Epidemiological Board FY 1970. Fort Detrick, MD.
98. Warren, J. R., Spero, L., and Metzger, J. F. (1974) The pH dependence of enterotoxin polymerization by formaldehyde. *Biochim. Biophys. Acta* **365(2),** 434–438.
99. Warren, J. R., et al. (1975) Immunogenicity of formaldehyde-inactivated enterotoxins A and C1 of *Staphylococcus aureus. J. Infect. Dis.* **131(5),** 535–542.
100. Tseng, J., et al. (1993) Immunity and responses of circulating leukocytes and lymphocytes in monkeys to aerosolized staphylococcal enterotoxin B. *Infect. Immun.* **61(2),** 391–398.
101. Tseng, J., et al. (1995) Humoral immunity to aerosolized staphylococcal enterotoxin B (SEB), a superantigen, in monkeys vaccinated with SEB toxoid-containing microspheres. *Infect. Immun.* **63(8),** 2880–2885.
102. Lowell, G. H., et al. (1996) Immunogenicity and efficacy against lethal aerosol staphylococcal enterotoxin B challenge in monkeys by intramuscular and respiratory delivery of proteosome-toxoid vaccines. *Infect. Immun.* **64(11),** 4686–4693.
103. Lowell, G. H., et al. (1996) Intranasal and intramuscular proteosome-staphylococcal enterotoxin B (SEB) toxoid vaccines: immunogenicity and efficacy against lethal SEB intoxication in mice. *Infect. Immun.* **64(5),** 1706–1713.
104. Ulrich, R. G., Olson, M. A., and Bavari, S. (1998) Development of engineered vaccines effective against structurally related bacterial superantigens. *Vaccine* **16(19),** 1857–1864.
105. Ulrich, R. G., Bavari, S., and Olson, M. A. (1995) Staphylococcal enterotoxins A and B share a common structural motif for binding class II major histocompatibility complex molecules. *Nat. Struct. Biol.* **2(7),** 554–560.

106. Leder, L., et al. (1998) A mutational analysis of the binding of staphylococcal enterotoxins B and C3 to the T cell receptor beta chain and major histocompatibility complex class II. *J. Exp. Med.* **187(6),** 823–833.
107. Olson, M. A. and Cuff, L. (1997) Molecular docking of superantigens with class II major histocompatibility complex proteins. *J. Mol. Recognit.* **10(6),** 277–289.
108. Woody, M. A., et al. (1998) Differential immune responses to staphylococcal enterotoxin B mutations in a hydrophobic loop dominating the interface with major histocompatibility complex class II receptors. *J. Infect. Dis.* **177(4),** 1013–1022.
109. Krupka, H. I., et al. (2002) Structural basis for abrogated binding between staphylococcal enterotoxin A superantigen vaccine and MHC-IIalpha. *Protein Sci.* **11(3),** 642–651.
110. DaSilva, L., et al. (2002) Humanlike immune response of human leukocyte antigen-DR3 transgenic mice to staphylococcal enterotoxins: a novel model for superantigen vaccines. *J. Infect Dis.* **185(12),** 1754–1760.
111. Coffman, J. D., et al. (2002) Production and purification of a recombinant Staphylococcal enterotoxin B vaccine candidate expressed in *Escherichia coli. Protein Expr. Purif.* **24(2),** 302–312.
112. Bavari, S., Dyas, B., and Ulrich, R. G. (1996) Superantigen vaccines: a comparative study of genetically attenuated receptor-binding mutants of staphylococcal enterotoxin A. *J. Infect. Dis.* **174(2),** 338–345.
113. Nilsson, I. M., et al. (1999) Protection against *Staphylococcus aureus* sepsis by vaccination with recombinant staphylococcal enterotoxin A devoid of superantigenicity. *J. Infect. Dis.* **180(4),** 1370–1373.
114. Swietnicki, W., et al. (2003) Zinc Binding and dimerization of streptococcus pyogenes pyrogenic exotoxin C are not essential for T-cell stimulation. *J. Biol. Chem.* **278(11),** 9885–9895.
115. Krakauer, T. and Buckley, M. (2003) Doxycycline is anti-inflammatory and inhibits staphylococcal exotoxin-induced cytokines and chemokines. *Antimicrob. Agents Chemother.* **47(11),** 3630–3633.
116. Krakauer, T., Li, B. Q., and Young, H. A. (2001) The flavonoid baicalin inhibits superantigen-induced inflammatory cytokines and chemokines. *FEBS Lett.* **500(1-2),** 52–55.
117. Krakauer, T. (2001) Suppression of endotoxin- and staphylococcal exotoxin-induced cytokines and chemokines by a phospholipase C inhibitor in human peripheral blood mononuclear cells. *Clin. Diagn. Lab. Immunol.* **8(2),** 449–453.
118. Cope, A. C. (1946) Chapter 12: Ricin in Summary technical report of Division 9 on Chemical warfare and related problems: Parts I-II. National Defense Research Committee, Office of Scientific Research and Development, Washington DC, pp. 179–203.
119. Knight, B. (1979) Ricin—a potent homicidal poison. *Br. Med. J.* **1(6159),** 350, 351.
120. Hewetson, J. F., et al. (1993) Protection of mice from inhaled ricin by vaccination with ricin or by passive treatment with heterologous antibody. *Vaccine* **11(7),** 743–746.
121. Griffiths, G. D., et al. (1996) The inhalation toxicology of the castor bean toxin, ricin, and protection by vaccination. *J. Defense Sci.* **1(2),** 227–235.
122. Crompton, R. and Gall, D. (1980) Georgi Markov—death in a pellet. *Med. Leg. J.* **48(2),** 51–62.
123. Franz, D. R. and Jaax, N. K. (1997) Ricin toxin, in *Medical Aspects of Chemical and Biological Warfare* (Sidell, F. R., Takafuji, E. T., and Franz, D. R., eds.), Office of the Surgeon General, Department of the Army, United States of America, Washington, D.C., pp. 631–642.
124. Robertus, J. (1991) The structure and action of ricin, a cytotoxic *N*-glycosidase. *Semin. Cell Biol.* **2(1),** 23–30.
125. Lord, J. M., Hartley, M. R., and Roberts, L. M. (1991) Ribosome inactivating proteins of plants. *Semin. Cell Biol.* **2(1),** 15–22.

126. Obrig, T. G. (1994) Toxins that inhibit host protein synthesis. *Methods Enzymol.* **235,** 647–656.
127. Balint, G. A. (1974) Ricin: the toxic protein of castor oil seeds. *Toxicology* **2(1),** 77–102.
128. Brugsch, H. G. (1960) Toxic hazards: The castor bean. *Mass. Med. Soc.* **262(1039-1040).**
129. Wilhelmsen, C. L. and Pitt, M. L. (1996) Lesions of acute inhaled lethal ricin intoxication in rhesus monkeys. *Vet. Pathol.* **33(3),** 296–302.
130. Griffiths, G. D., Phillips, G. J., and Bailey, S. C. (1999) Comparison of the quality of protection elicited by toxoid and peptide liposomal vaccine formulations against ricin as assessed by markers of inflammation. *Vàccine* **17(20-21),** 2562–2568.
131. Ghetie, V. and Vitetta, E. (1994) Immunotoxins in the therapy of cancer: from bench to clinic. *Pharmacol. Ther.* **63(3),** 209–234.
132. Vitetta, E. S., Thorpe, P. E., and Uhr, J. W. (1993) Immunotoxins: magic bullets or misguided missiles? *Trends Pharmacol. Sci.* **14(5),** 148–154.
133. Soler-Rodriguez, A. M., et al. (1992) The toxicity of chemically deglycosylated ricin A-chain in mice. *Int. J. Immunopharmacol.* **14(2),** 281–291.
134. Lord, J. M., et al. (1987) Ricin: cytotoxicity, biosynthesis and use in immunoconjugates. *Prog. Med. Chem.* **24,** 1–28.
135. Lemley, P. V. and Creasia, D. A. (1995) Vaccine against ricin toxin, in U. S. Patent & Trademark Office. United States of America, Secretary of the Army, Washington, DC.
136. Lemley, P. V. and Wright, D. C. (1992) Mice are actively immunized after passive monoclonal antibody prophylaxis and ricin toxin challenge. *Immunology* **76(3),** 511–513.
137. Aboud-Pirak, E., et al. (1993) Identification of a neutralizing epitope on ricin a chain and application of its 3D structure to design peptide vaccines that protect against ricin intoxication, in 1993 Medical Defense Bioscience Review. U. S. Army Medical Research & Materiel Command, Baltimore, MD.
138. Griffiths, G. D., et al. (1998) Local and systemic responses against ricin toxin promoted by toxoid or peptide vaccines alone or in liposomal formulations. *Vaccine* **16(5),** 530–535.
139. Smallshaw, J. E., et al. (2002) A novel recombinant vaccine which protects mice against ricin intoxication. *Vaccine* **20(27-28),** 3422–3427.
140. Olson, M. A. (1997) Ricin A-chain structural determinant for binding substrate analogues: a molecular dynamics simulation analysis. *Proteins* **27(1),** 80–95.
141. Olson, M. A. and Cuff, L. (1999) Free energy determinants of binding the rRNA substrate and small ligands to ricin A-chain. *Biophys. J.* **76(1 Pt 1),** 28–39.
142. Olson, M. A. (2001) Electrostatic effects on the free-energy balance in folding a ribosome-inactivating protein. *Biophys. Chem.* **91(3),** 219–229.
143. Tanaka, K. S., et al. (2001) Ricin A-chain inhibitors resembling the oxacarbenium ion transition state. *Biochemistry* **40(23),** 6845–6851.
144. Miller, D. J., et al. (2002) Structure-based design and characterization of novel platforms for ricin and shiga toxin inhibition. *J. Med. Chem.* **45(1),** 90–98.
145. Hopkins, A. L. and Groom, C. R. (2002) The druggable genome. *Nat. Rev. Drug Discov.* **1(9),** 727–730.
146. Finkel, E. (2001) Australia. Engineered mouse virus spurs bioweapon fears. *Science* **291(5504),** 585.
147. Landgraf, R., et al. (1998) Cytotoxicity and specificity of directed toxins composed of diphtheria toxin and the EGF-like domain of heregulin beta1. *Biochemistry* **37(9),** 3220–3228.
148. vanderSpek, J. C. and Murphy, J. R. (2000) Fusion protein toxins based on diphtheria toxin: selective targeting of growth factor receptors of eukaryotic cells. *Methods Enzymol.* **327,** 239–249.
149. Francis, J. W., et al. (2000) Enhancement of diphtheria toxin potency by replacement of the receptor binding domain with tetanus toxin C-fragment: a potential vector for delivering heterologous proteins to neurons. *J. Neurochem.* **74(6),** 2528–2536.

150. Fisher, C. E., et al. (1996) Genetic construction and properties of a diphtheria toxin-related substance P fusion protein: in vitro destruction of cells bearing substance P receptors. *Proc. Natl. Acad. Sci. USA* **93(14),** 7341–7345.
151. Arora, N., et al. (1994) Cytotoxic effects of a chimeric protein consisting of tetanus toxin light chain and anthrax toxin lethal factor in non-neuronal cells. *J. Biol. Chem.* **269(42),** 26,165–26,171.
152. Arora, N. and Leppla, S. H. (1994) Fusions of anthrax toxin lethal factor with shiga toxin and diphtheria toxin enzymatic domains are toxic to mammalian cells. *Infect. Immun.* **62(11),** 4955–4961.
153. Walev, I., et al. (2001) Delivery of proteins into living cells by reversible membrane permeabilization with streptolysin-*O*. *Proc. Natl. Acad. Sci USA* **98(6),** 3185–3190.
154. Ohno, M., et al. (1998) Molecular evolution of snake toxins: is the functional diversity of snake toxins associated with a mechanism of accelerated evolution? *Prog. Nucleic Acid Res. Mol. Biol.* **59,** 307–364.
155. Le Du, M. H., et al. (2000) Stability of a structural scaffold upon activity transfer: X-ray structure of a three fingers chimeric protein. *J. Mol. Biol.* **296(4),** 1017–1026.
156. Harel, M., et al. (1995) Crystal structure of an acetylcholinesterase-fasciculin complex: interaction of a three-fingered toxin from snake venom with its target. *Structure* **3(12),** 1355–1366.
157. Meves, H., Simard, J. M., and Watt, D. D. (1986) Interactions of scorpion toxins with the sodium channel, in *Tetrodotoxin, Saxitoxin, and The Molecular Biology of the Sodium Channel* (Yao, C. Y. and Levinson, S. R., eds.), The New York Academy of Sciences, New York, NY, pp. 113–132.
158. Zilberberg, N., et al. (1996) Functional expression and genetic alteration of an alpha scorpion neurotoxin. *Biochemistry* **35(31),** 10,215–10,222.
159. Bouhaouala-Zahar, B., et al. (2000) A chimeric scorpion alpha-toxin displays *de novo* electrophysiological properties similar to those of alpha-like toxins. *Eur. J. Biochem.* **269(12),** 2831–2841.
160. Olivera, B. M., et al. (1985) Peptide neurotoxins from fish-hunting cone snails. *Science* **230(4732),** 1338–1343.
161. Broomfield, C. A., Lockridge, O., and Millard, C. B. (1999) Protein engineering of a human enzyme that hydrolyzes V and G nerve agents: design, construction and characterization. *Chem. Biol. Interact.* **119–120,** 413–418.
162. Sun, H., et al. (2002) Cocaine metabolism accelerated by a re-engineered human butyrylcholinesterase. *J. Pharmacol. Exp. Ther.* **302(2),** 710–716.
163. Lacy, D. B. and Stevens, R. C. (1998) Unraveling the structures and modes of action of bacterial toxins. *Curr. Opin. Struct. Biol.* **8(6),** 778–784.
164. Gerstein, M. (2000) Integrative database analysis in structural genomics. *Nat. Struct. Biol.* **7(Suppl.),** 960–963.
165. Gerstein, M., et al. (2003) Structural genomics: current progress. *Science* **299(5613),** 1663.